Denitrifying haloarchaea: sources and sinks of nitrogenous gases

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Abstract:

Haloarchaea thrive under saline and hypersaline conditions and often dominate microbial communities in saltmarshes, salted lakes/soils, and some oceanic areas. Some of the predominant species show denitrifying capabilities, although it remains unclear whether they are complete or partial denitrifiers. As complete denitrifiers, they could play important roles buffering ecosystems in which nitrate and nitrite appear as contaminants. However, partial denitrifying haloarchaea could contribute to the emission of nitrogenous gasses, thus acting as drivers of climate change and ozone depletion. In this review, we summarise some recent results on denitrification in haloarchaea, discuss the environmental implications and outline possible applications in mitigation. Finally, we list questions to be addressed in the near future, facilitating increased understanding of the role of these organisms in N turnover in arid and hypersaline environments.

Keywords: denitrification, nitric oxide, nitrous oxide, haloarchaea, greenhouse gas, climate change.

Introduction

Anthropogenic activities are major drivers of climate forcing (Estrada et al., 2017; Medhaug et al., 2017), with implications for terrestrial aridity and salinity in continental and oceanic ecosystems. Drylands currently cover about 41% of Earth's land surface and there is concern that the predicted increase in aridity due to climate change could disrupt the cycles of nutrients like carbon (Delgado-Baquerizo et al., 2013) (Figure 1). Saline and hypersaline environments are concomitant with arid and semi-arid areas across the globe. Salinization affects many different aspects of life. It alters the chemical composition of natural water resources, degrading the quality of water supply to all sectors, which in turn leads to a range of health issues, loss of fertile soil and collapse of agri- and aqua-cultural industries. Moreover, it contributes to loss of biodiversity and taxonomic replacement by halotolerant/halophilic species (Williams, 2001). Some geographical areas such as the Mediterranean countries, Australia, North Africa and coastal areas in North and South America are highly affected by these phenomena (Figure 1), but currently there are no accurate data of the prevalence and total area of saline/hypersaline environments. As an example, some best estimates of the area of saltmarshes range from 2.2 to 40 Mha (Pendleton et al., 2012). Considering that saltmarshes are not the predominant hypersaline ecosystems, the extensiveness of these environments is in fact significantly larger and expected to increase due to climate change. Thus, the spread and biology of such systems are interesting from a global perspective.

Recent studies addressing the role of microorganisms in arid-semiarid soils reveal that microbial communities drive the resistance of ecosystem multi-functionality to environmental change (Delgado-Baquerizo et al., 2017). Thus, it stands to reason that the composition of microbial communities is important for buffering effects of climate change in drylands worldwide. Hypersaline environments are inhabited by microbes, mainly halophilic archaea, thriving under high salt concentration. Although haloarchaea were first described in the mid-1950s, the knowledge about their metabolism, physiology and general role in their ecosystems

53 is scarce. One trait that makes some haloarchaea particularly interesting from an ecological
54 perspective is their ability to sustain respiratory growth under hypoxic or anoxic conditions
55 through denitrification. Some are full-fledged denitrifiers, capable of reducing nitrate to N_2 via
56 nitrite, NO and N_2O (Zumft 1997). Such organisms effectively remove NOx^- from soils and water
57 and may act as both sources and sinks of NO and N_2O, depending on their regulatory set-up.
58 Others carry out only parts of the process, and may act as net sources of gaseous N-oxides,
59 perhaps most importantly N_2O, contributing to global warming and ozone depletion
60 (Ravishankara et al., 2009). In light of the increasing input rates of nitrate, nitrite and ammonium
61 in arid and semi-arid areas from agriculture (Martínez-Espinosa et al., 2011), the significance of
62 denitrifying haloarchaea in such systems is worth exploring.
63 This work summarised new insights into the contribution of denitrifying haloarchaea to
64 climate change and their potential use as sinks for nitrogenous gases, or as key players in
65 bioremediation strategies.
66
67 **Saline environments and their inhabitants**
68 Saline and hypersaline environments are found worldwide and include genuine
69 ecosystems such as the Dead Sea (Israel-Palestine), Great Salt Lake (USA) and coastal marshes,
70 or inland/coastal ponds from which salt has been extracted for many centuries (Zafrilla et al.,
71 2010). Hypersaline environments can be classified into two categories: Thalassohaline, i.e.
72 originating from seawater with Na^+ and Cl^- as the predominant ions, and Athalassohaline, which
73 display variable ionic compositions influenced by the area where the pond develops (Grant,
74 2004). The biodiversity of these environments is higher than initially expected, but obviously
75 lower than that of non-saline ecosystems. Among the main representative organisms inhabiting
76 these environments are *Artemia* (shrimp), *Dunaliella* (unicellular algae), *Salinibacter* (red
77 bacteria), and several genera from the Archaea Domain, commonly named Haloarchaea,
78 belonging to the families *Haloferacaceae* and *Halobacteriaceae* (Figure 2). The family

Halobacteriaceae consists of 11 genera: *Halobacterium* (the type genus), *Haladaptatus*, *Halalkalicoccus*, *Halarchaeum*, *Haloarchaeobius*, *Halomarina*, *Halorubellus*, *Halorussus*, *Halovenus*, *Natronoarchaeum*, and *Salarchaeum*. Before 2015, *Halobacteriaceae* was the only family in the class *Halobacteria*. In 2015, the family was split into three orders and six families based on phylogenomic analyses (Oren et al., 2017). Hence, the other important family grouping of haloarchaea is the family *Haloferacaceae,* which is comprised of seven genera: *Haloferax*, *Halobellus*, *Halogeometricum* (including two species previously assigned to the genus *Halosarcina*), *Halogranum*, *Halopelagius*, *Haloplanus*, and *Haloquadratum*. Members of these two families are neutrophilic, halophilic or extremely halophilic Archaea, mostly red-pink pigmented and generally leading an aerobic life style (Gupta et al., 2016).

It has been extensively reported that haloarchaea constitute the dominant microbial populations in habitats where the NaCl concentration ranges from 20% (w/v) up to halite saturation (<32% (w/v) (Oren, 2002). Therefore, haloarchaea may contribute significantly to the global biogeochemical cycles through their activity in these environments. Representatives from the *Haloferax, Haloarcula* and *Natronomonas* genera are the best described at the time of writing this review. Some of these species carry genes encoding one or several of the metal containing enzymes involved in denitrification, but further studies *in vitro* and *in situ* are required to assess their contribution to the homeostasis of the ecosystems they inhabit.

Denitrification, bacteria vs archaea

All organisms require nitrogen as an essential building block in macromolecules such as nucleic acids and proteins. The reserves of reactive nitrogen in the biosphere originates from, and is returned to, our atmosphere where it chiefly exists as chemically inert N_2. Microbes are major drivers of the global nitrogen cycle, harnessing nitrogen in all its redox states, from the most reduced (NH_3; −3), to the most oxidized state (NO_3^-; +5) (Bothe et al., 2007). The past century has seen a dramatically increased N-input to the biosphere, and anthropogenic activities

currently results in a doubling of the annual global N-budget, chiefly through the production and application of artificial fertilizers (Schlesinger 2009). This has resulted in an escalation of the global N-cycle and increasing emissions of NO, N_2O and N_2 in proportions controlled by the physiology of the microbes involved. Denitrification is the major biological source of N_2O to the atmosphere (Schlesinger 2009), and the only known sink, underscoring the inherent importance of studying the dominating denitrifiers in each environment. The biochemistry and physiology of denitrification have been extensively studied in a limited selection of prokaryotes, all of which belong to the Bacteria domain, and most of which belong to the proteobacteria. The complete reduction of nitrate to N_2, is driven by metal containing enzymes: nitrate-(Nar), nitrite-(Nir), nitric oxide-(Nor) and nitrous oxide-(Nos) reductases, which replace the terminal oxidases of the aerobic electron transport chain (Figure 3) (Lledó *et al.,* 2004; Richardson and Watmough, 1999). Each step, with the exception of the final reduction of N_2O to N_2, can be driven by at least two alternative, functionally equal redox enzymes, and one organism may carry one or more variants. Nitrate may be reduced to nitrite by NarGHI or NapAB type enzymes (Gates et al., 2011). The nitrite produced is further reduced to nitric oxide either by the Cu-enzyme NirK or the structurally unrelated cytochrome cd1 type nitrite reductase NirS. Nitric oxide is reduced to nitrous oxide by qNor, cNor or qCuANor-type enzymes (Al-Attar and de Vries 2015), and finally nitrous oxide is reduced to dinitrogen by the unique NosZ (Zumf 1997). The catalytic sites of all these enzymes, except Nar, face the positive side of the cytoplasmic membrane, and in gram negative bacteria, Nir and NosZ are soluble in the periplasm (Kraft et al., 2011; Verbaendert et al., 2011). Less is known of Archaea, but recent findings have shown that some of the most abundant haloarchaeal species show denitrification capabilities (Torregrosa-Crespo et al., 2016). Many of the studies of denitrification in haloarchaea have been done in *Haloarcula marismortui* and representatives of the *Haloferax* genus. Purification and characterization of the enzymes catalysing the two first reactions in the *Haloarcula* and *Haloferax* genera revealed that nitrate is reduced to nitrite by a periplasmic NarGHI type nitrate reductase (pNar) (Martínez-Espinosa et

al., 2007), and nitrite is further reduced to nitric oxide by the Cu-enzyme NirK (Esclapez et al., 2013; Martínez-Espinosa et al., 2007; Torregrosa-Crespo et al., 2016). Analyses of the haloarchaea available genomes show that genes coding for the NirS type enzyme are not present. There are no biochemical studies on neither Nor or Nos enzymes from haloarchaea at the time of writing this work. However, a comparative analysis of multiple haloarchaeal genomes suggests that the quinone-dependent respiratory nitric oxide reductase is the key enzyme for nitric oxide reduction in haloarchaea (Torregrosa-Crespo et al., 2017). Biochemical studies on nitrous oxide reductases in haloarchaea have not been conducted yet, but preliminary data suggest that, as in bacteria, the last reaction of the denitrification is catalysed by a NosZ-like protein. Thus, it appears that denitrification in haloarchaea bear similarity to variants seen in bacteria (Figure 3).

Haloarchaeal denitrifiers as N-oxide sources and sinks, potential biotechnological applications.

The identification and characterization of new microbial taxa involve the analysis of morphological features (cell morphology and motility) as well as metabolic and physiologic capabilities such as redox enzymes activities, use of several carbon sources, etc. The ability to denitrify is usually tested by checking nitrate reduction under anaerobic conditions, but further analyses of N-oxide production is often lacking or somewhat crude. Thus, many species are characterised as denitrifiers when in fact they are just partial denitrifiers. In other cases, the strains are able to produce nitrogenous gases not from nitrate but from nitrite, or they may be able to carry out only the final steps of denitrification (Lycus et al., 2017). Consequently, this characterisation is not always accurate.

At the time of writing this review, several well-known haloarchaeal species have been described as denitrifiers. Many of them group into the *Haloferax*, *Haloarcula* and *Natrinema* genera (Feng et al., 2012; Torregrosa-Crespo et al., 2016). However, it remains unclear whether

157 or not they are complete denitrifiers. Some preliminary studies suggest that at least *Haloferax*
158 *mediterranei* could be a complete denitrifier (Torregrosa-Crespo et al. 2016). This organism is
159 quite common in moderate and hypersaline environments (including water bodies and soils). In
160 this context, i.e. high salt concentrations, relatively high temperatures (around 18ºC in winter
161 and up to 45 ºC in summer) and low oxygen solubility, *Haloferax mediterranei* is likely to be a
162 highly relevant player in the reduction of nitrogen oxyanions to gaseous N-oxides and N_2, thus
163 buffering ecosystems in which nitrate and nitrite appear as contaminants. This makes *Haloferax*
164 *mediterranei* an interesting subject for further study and a candidate for future application in
165 nitrogen removal from saline and hypersaline environments (Nájera et al., 2012; Torregrosa-
166 Crespo et al., 2016)

167 Denitrifying microorganisms, and in particular extremophiles able to denitrify, have
168 caught the interest of research communities searching for new strategies for soil and
169 wastewater bioremediation (Martínez-Espinosa et al., 2015; Nájera et al., 2012; Torregrosa-
170 Crespo, et al., 2016). However, we must be careful when we set out to pick our candidates. Life
171 sciences have seen a fantastic technological evolution and recent advances in sequencing
172 technologies allow us to sequence genomes, meta-genomes and -transcriptomes of microbial
173 communities “in the blink of an eye”. These are powerful tools, but in our search for denitrifiers,
174 we need to be aware of their limitations. At present, the genotype of a denitrifier, be it full-
175 fledged or partial, only informs us of its potential (Lycus et al., 2017; Roco et al., 2017). In order
176 to ascertain whether the organism is likely to be a source or sink of environmentally harmful
177 intermediates, refined phenotypic testing is required. Previous studies of denitrifying bacteria
178 have revealed a number of regulatory traits that directly affect the organisms’ propensities for
179 NO_x accumulation. Some full-fledged denitrifiers have been shown to express functional N_2O
180 reductase as a response to hypoxia, and before “upstream” enzymes, thus acting as net sinks of
181 N_2O (Qu et al., 2016). Others appear to poorly control the relative expression of denitrification
182 enzymes, resulting in the release of substantial amounts of nitrite and gaseous intermediates

(Lycus et al., 2017; Roco et al., 2017). Yet others are unable to reduce externally supplied N_2O although they carry functional N_2O reductase, thus acting as net sources, albeit weak, of N_2O (Thomson et al., 2012). These and other phenotypic traits must be taken into consideration when evaluating potential candidates for application in (N) bioremediation.

Conclusions

More than 50 years of research on extremophiles have resulted in a remarkable improvement of the knowledge about their molecular biology, biochemistry, physiology, microecology and general structural features. Nevertheless, their role in natural environments and their potential contribution on biogeochemical cycles remains unknown. Recent studies on denitrifying haloarchaea suggest that complete denitrifiers are important for nitrate reduction to dinitrogen in saline/hypersaline soils and water bodies, and can act as sinks for nitrogenous gases in these ecosystems. However, laboratory scale experiments with haloarchaea displaying partial denitrification suggest that they could contribute significantly to NO and N_2O emissions, consequently contributing to ozone depletion and climate change. Macro-scale experiments and experiments *in situ* must be conducted in order to address the myriad of open questions related to haloarchaea and their role in climate change and ecosystems buffering;

- How abundant are complete and partial denitrifying haloarchaea in their ecosystems?
- How efficient are the individual denitrification enzymes?
- How effective is the regulatory set-up of haloarchaeal denitrifiers with respect to minimising the release of intermediates from their environment?
- How significant is the emission of nitrogenous gasses by partial denitrifying haloarchaea in their natural ecosystems?

- Is there a typical denitrification genotype in hypersaline environments, in terms of the type of gene clusters carried by the organisms, e.g. *nar* vs *nap*, *nirK* vs *nirS* etc.?

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Legends to figures:

Figure 1: A) Map of dry lands geomorphology (Adapted from: http://www.millenniumassessment.org/en/About.html); B) Annual mean of the sea surface salinity distribution (Adapted from World Ocean Atlas, 2005; http://www.salinityremotesensing.ifremer.fr/sea-surface-salinity/salinity-distribution-at-the-ocean-Surface)

Figure 2: Pictures of some organisms that are typically strongly represented in hypersaline environments like saltmarshes or salted ponds. A) The brine shrimp *Artemia salina* (natural food for flamingos, for instance; B) The micro-algae *Dunaliella salina* (from which carotenoids are obtained at large scale), C) *Haloferax volcanii* colonies (haloarchaea) D) Electronic microscopy picture showing *Haloferax mediterranei* cells (the white granules correspond to PHA accumulations). In fact, the red colour associated with hypersaline lakes and ponds is mainly due to the pigmentation of halophilic archaea and the eukaryote *Dunaliella* (Mancinelli, 2005).

Figure 3: The denitrification process: summary of the reactions and enzymes involved. Black names correspond to the enzymes catalasing the reactions in Bacteria, whilst blue names correspond to the enzymes involved in denitrification in haloarchaea.

386

387 **Figure 1:**

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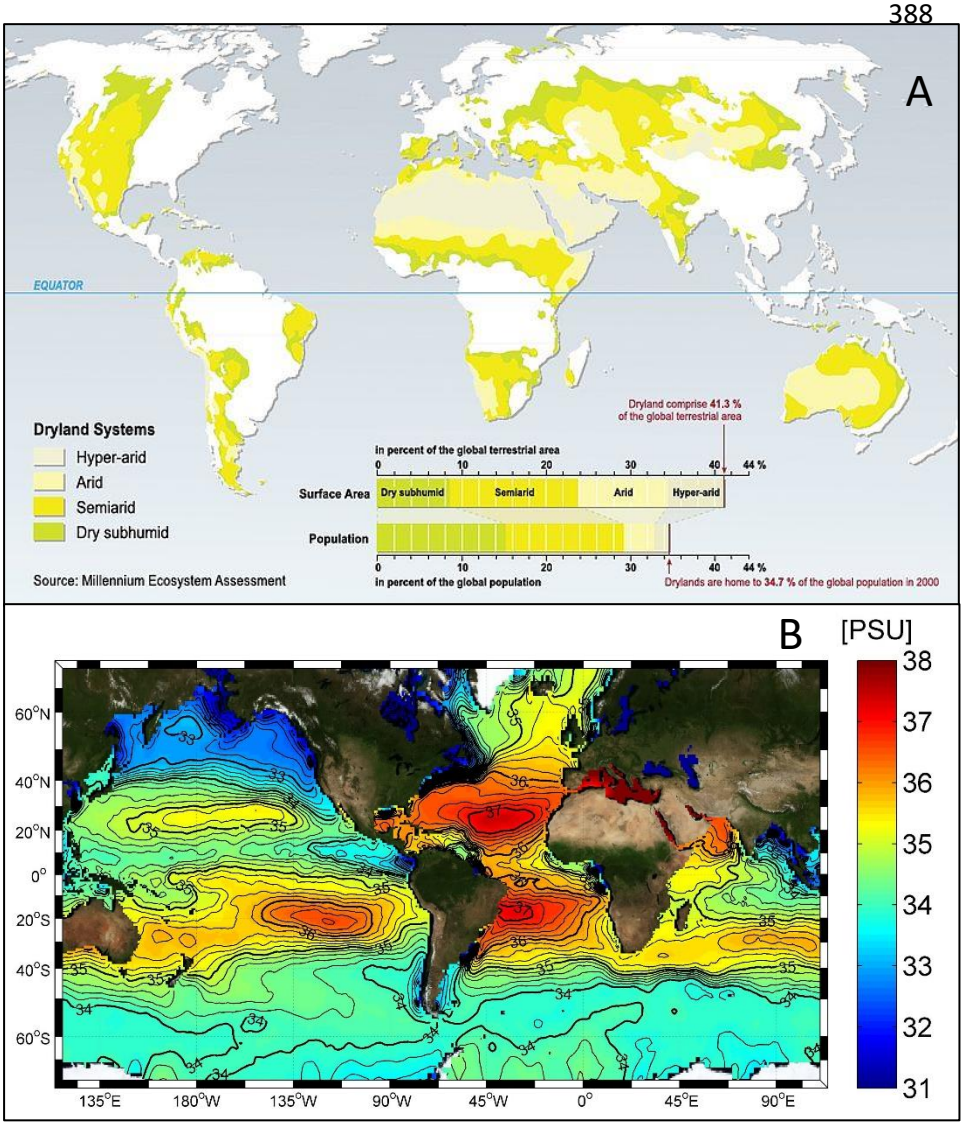

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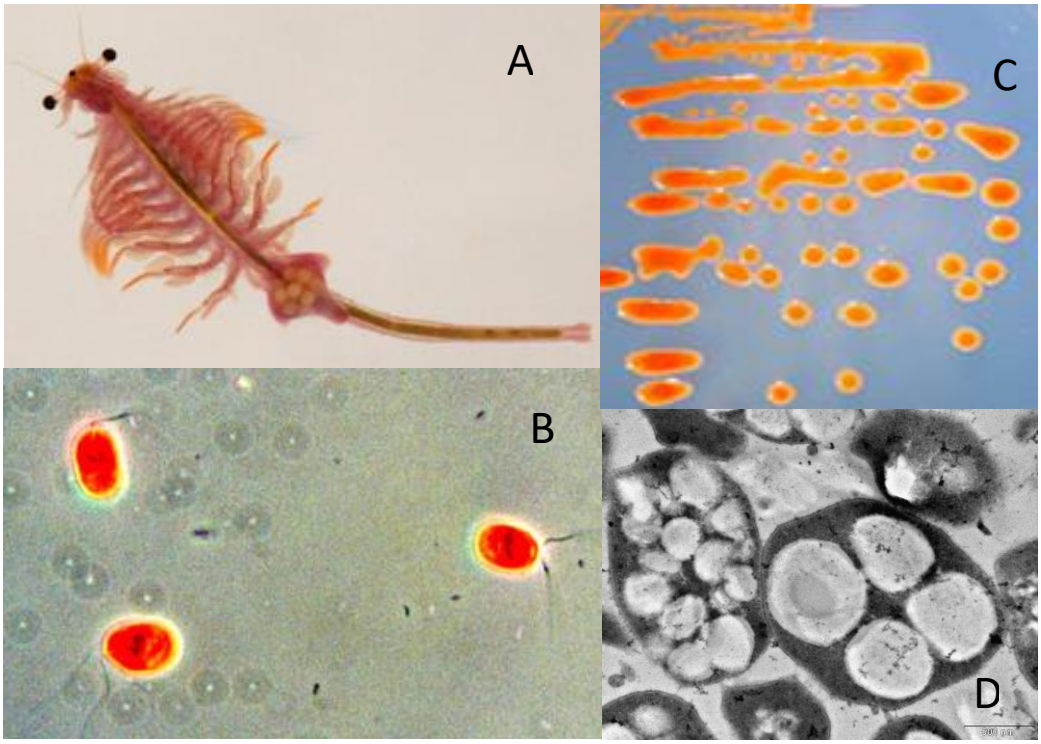
Figure 2:

Brine shrimp (*Artemia salina*)

Haloarchaea (*Haloferax volcanii*)



Micro-algae (*Dunaliella salina*)

Haloarchaea (*Haloferax mediterranei* cells)

Figure 3: